AK

CORRECTED VERSION*

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 :
C07K 15/00, C12N 15/15
A61K 37/64, C07K 13/00

A3

(11) International Publication Number: WO 94/16073

(43) International Publication Date: 21 July 1994 (21.07.94)

(21) International Application Number: PCT/US94/00247

(22) International Filing Date: 7 January 1994 (07.01.94)

(30) Priority Data:
08/002,202 8 January 1993 (08.01.93) US

(60) Parent Application or Grant
(63) Related by Continuation
US 08/002,202 (CIP)
Filed on 8 January 1993 (08.01.93)

(71) Applicant *(for all designated States except US)*: STATE OF OREGON, acting by and through THE OREGON STATE BOARD OF HIGHER EDUCATION ON BEHALF OF THE OREGON HEALTH SCIENCES UNIVERSITY [US/US]; 3181 S.W. Sam Jackson Park Road, Portland, OR 97201-3098 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: THOMAS, Gary [US/US]; 5210 Wichita, Tualatin, OR 97062 (US). ANDERSON, Eric, D. [US/US]; 4512 S.E. Windsor, Portland, OR 97202 (US). THOMAS, Laurel [US/US]; 5210 Wichita, Tualatin, OR 97062 (US). HAYFLICK, Joel, S. [US/US]; 7003 54th Avenue S.E., Seattle, WA 98115 (US). NELSON, Jay [US/US]; 21067 S.W. Meadow Way, Tualatin, OR 97062 (US). STENGLEN, Stephan, G. [DE/US]; 163 S.W. Bancroft Court, Portland, OR 97201 (US).

(74) Agent: NOONAN, Kevin, E.; Allegretti & Witcoff, Ltd., Ten South Wacker Drive, Chicago, IL 60606 (US).

(81) Designated States: AU, CA, FI, JP, NO, US, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(88) Date of publication of the international search report:
24 November 1994 (24.11.94)

(54) Title: METHODS AND REAGENTS FOR INHIBITING FURIN ENDOPROTEASE

(57) Abstract

This invention relates to methods and reagents for inhibiting furin endoprotease activity and specifically for inhibiting furin endoprotease-mediated maturation of bioactive proteins *in vivo* and *in vitro*. The invention specifically provides proteins capable of inhibiting furin endoprotease activity. Particularly provided are α_1-antitrypsin variants that specifically inhibit furin endoprotease activity. Methods for using furin endoprotease inhibition to attenuate or prevent viral protein maturation, and thereby alleviate viral infections, are provided. Also provided are methods for using furin endoprotease inhibition to attenuate or prevent proteolytic processing of bacterial toxins, thereby alleviating bacterial infections. Methods are also provided to inhibit proteolytic processing of biologically active proteins and peptides. The invention also provides pharmaceutically acceptable compositions of therapeutically effective amounts of furin endoprotease inhibitors.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	GB	United Kingdom	MR	Mauritania
AU	Australia	GE	Georgia	MW	Malawi
BB	Barbados	GN	Guinea	NE	Niger
BE	Belgium	GR	Greece	NL	Netherlands
BF	Burkina Faso	HU	Hungary	NO	Norway
BG	Bulgaria	IE	Ireland	NZ	New Zealand
BJ	Benin	IT	Italy	PL	Poland
BR	Brazil	JP	Japan	PT	Portugal
BY	Belarus	KE	Kenya	RO	Romania
CA	Canada	KG	Kyrgystan	RU	Russian Federation
CF	Central African Republic	KP	Democratic People's Republic of Korea	SD	Sudan
CG	Congo			SE	Sweden
CH	Switzerland	KR	Republic of Korea	SI	Slovenia
CI	Côte d'Ivoire	KZ	Kazakhstan	SK	Slovakia
CM	Cameroon	LI	Liechtenstein	SN	Senegal
CN	China	LK	Sri Lanka	TD	Chad
CS	Czechoslovakia	LU	Luxembourg	TG	Togo
CZ	Czech Republic	LV	Latvia	TJ	Tajikistan
DE	Germany	MC	Monaco	TT	Trinidad and Tobago
DK	Denmark	MD	Republic of Moldova	UA	Ukraine
ES	Spain	MG	Madagascar	US	United States of America
FI	Finland	ML	Mali	UZ	Uzbekistan
FR	France	MN	Mongolia	VN	Viet Nam
GA	Gabon				

METHODS AND REAGENTS FOR INHIBITING FURIN ENDOPROTEASE

BACKGROUND OF THE INVENTION

This invention was made with government support under DK44629 and DK37274 from the National Institutes of Health. The government has certain rights in the invention.

1. Field of the Invention

This invention relates to endoproteases, particularly a novel endoprotease termed furin endoprotease. The invention also relates to inhibitors of furin endoprotease activity. In particular, the invention relates to novel variants of α_1-antitrypsin that specifically inhibit furin endoprotease activity. The invention also provides methods for using such inhibitors to attenuate or prevent biological proteolytic maturation of bioactive proteins and peptides *in vivo* and *in vitro*, in particular viral proteins and bacterial toxins. Therapeutic methods and pharmaceutical compositions of such inhibitors are also provided directed towards the alleviation and treatment of disease having microbiological etiology.

2. Background of the Related Art

Most biologically active peptides and proteins are synthesized initially as larger, inactive precursor proteins that are endoproteolytically cleaved during transit through the secretory pathway in the Golgi apparatus in cells expressing such proteins (*see* Barr, 1991, Cell 66: 1-3 for review). This system comprises an important common mechanism required for synthesis of biologically active proteins and peptides in yeast (Fuller *et al.*, 1988, Ann. Rev. Physiol. 50: 345-362), invertebrates (Scheller *et al.*, 1983, Cell 32: 7-22) and mammalian cells (Sossin *et al.*, 1989, Neuron 2: 1407-1417). Examples of proteins produced *in vivo* by exocytotic transport through the Golgi are precursors of peptide hormones, neuropeptides, growth factors, coagulation factors, serum albumin, cell surface receptors, and adhesion molecules.

Morrison *et al.*, 1985, J. Virol. 53: 851-857 disclose that F protein of

Newcastle disease virus is processed through the exocytotic transport pathway in infected cells.

Perez & Hunter, 1987, J. Virol. 61: 1609-1614 disclose that the Rous sarcoma virus (RSV) glycoprotein is processed through the exocytotic transport pathway in infected cells.

Yamada *et al.*, 1988, Virology 165: 268-273 disclose that F protein of mumps virus is processed through the exocytotic transport pathway in infected cells.

Randolph *et al.*, 1990, Virology 174: 450-458 disclose that the prM protein of flaviviruses is processed through the exocytotic transport pathway in infected cells.

A common structural feature of molecules processed through the exocytotic transport pathway is the presence of basic residues or pairs of basic residues at the proteolytic processing site in the molecule. Examples include serum factors (Factor IX; Bentley *et al.*, 1986, Cell 45: 343-348; proalbumin; Knowles *et al.*, 1980, Science 209: 497-499; pro-von Willibrand factor; Bonthron *et al.*, 1986, Nature 324: 270-273), viral polyproteins (human immunodeficiency virus (HIV) gp160; McCune *et al.*, 1988, Cell 53: 55-67; RSV envelope protein; Perez & Hunter, 1987, J. Virol. 61: 1609-1614; yellow fever virus protein; Rice *et al.*, 1985, Science 229: 726-733; measles virus protein; Richardson *et al.*, 1986, Virology 155: 508-523; mumps virus protein; Waxham *et al.*, 1987, Virology 159: 381-389; human cytomegalovirus protein; Spaete *et al.*, 1990, J. Virol. 64: 2922-2931; varicella zooster virus protein; Keller *et al.*, 1986, Virology 152: 181-191), growth factors (pre-protransforming growth factor β; Gentry *et al.*, 1988, Molec. Cell. Biol. 8: 4162-4168; epidermal growth factor; Gray *et al.*, 1983, Nature 303: 722-725; pro-β-nerve growth factor (NGF); Edwards *et al.*, 1988, Molec. Cell Biol. 8: 2456-2464), receptors (insulin receptor; Yoshimasa *et al.*, 1988, Science 240: 784-787); and bacterial toxins [*see* Stephen & Pietrowski, 1986, Bacterial Toxins, 2d ed. (Amer. Soc. Microbiol. Washington, D.C.) for review; anthrax toxin; Singh *et al.*, 1989, J. Biol. Chem. 264: 11099-11102]. The proteolytic

processing site has been identified in some of these molecules.

Berger & Shooter, 1977, Proc. Natl. Acad. Sci. USA 74: 3647-3651 disclose the sequence -RSKR- at the proteolytic processing site of pro-β-NGF.

Bentley *et al.*, 1986, *ibid.* disclose the sequence -RPKR- at the proteolytic processing site of the blood coagulation factor protein Factor IX.

McCune *et al.*, 1988, *ibid.*, disclose the sequence -REKR- at the proteolytic processing site of HIV gp160.

Clepak *et al.*, 1988, Biochem. Biophys. Res. Comm. 157: 747-754 disclose the sequence -RVRR- at the proteolytic processing site of diphtheria toxin.

Vey *et al.*, 1992, Virology 188: 408-413 disclose the sequence -RX(R/K)R- at the proteolytic processing site of influenza hemagglutinin.

Ogata *et al.*, 1990, J. Biol. Chem. 265: 20678-20685 disclose the sequence -RSKR- at the proteolytic processing site of *Pseudomonas* exotoxin A.

Klimpel *et al.*, 1992, Proc. Natl. Acad. Sci. USA 89: 10277-10281 disclose the sequence -RX(R/K)R- at the proteolytic processing site of anthrax protective antigen.

Recently, an endoprotease termed furin has been isolated that specifically recognizes the recognition sequence of proteins processed through the exocytotic secretory pathway (Wise *et al.*, 1990, Proc. Natl. Acad. Sci. USA 87: 9378-9382; Bresnahan *et al.*, 1990, J. Cell Biol. 111: 2851-2859). This endoprotease is a subtilisin-related, calcium-dependent serine protease (Bresnahan *et al.*, *ibid.*). A complementary DNA copy of the mRNA encoding this endoprotease has been isolated (Wise *et al.*, *ibid.*) and sequenced (van den Ouweland *et al.*, 1992, Nucleic Acids Res. 18: 664) and expressed in heterologous cells (Bresnahan *et al.*, *ibid.*). These studies have shown furin to be expressed as a doublet of 96 and 90 kilodaltons (kD) in size, ubiquitously expressed as a 4.5 kilobase (kb) mRNA, and localized by fluorescence immunohistochemistry to the Golgi apparatus of cells expressing this endoprotease (Bresnahan *et al.*, *ibid.*). Furin has been shown to be capable of proteolytically cleaving a number of exocytotically processed proteins.

Bresnahan *et al.*, *ibid.*, disclose furin-mediated cleavage of pro-β-NGF.

Wise *et al.*, *ibid.*, disclose furin-mediated cleavage of pro-von Willibrand factor and complement factor C3.

Hosaka *et al.*, 1991, J. Biol. Chem. 266: 12127-12130 disclose furin-mediated cleavage of renin.

Steineke-Grober *et al.*, 1992, EMBO J. 11: 2407-2414 disclose furin-mediated cleavage of influenza hemagglutinin.

Klimpel *et al.*, 1992, Proc. Natl. Acad. Sci. USA 89: 10277-10281 disclose furin-mediated cleavage of anthrax protective antigen.

Molloy *et al.*, 1992, J. Biol. Chem 267: 16396-16402 disclose furin-mediated cleavage of anthrax protective antigen.

Klimpel *et al.*, 1992, Annual Meeting, Amer. Soc. Microbiol. Abst. B-32 disclose furin-mediated cleavage of diphtheria toxin.

Furin can be inhibited by specific peptidyl chloroalkylketones (Garten *et al.*, 1989, Virology 172: 25-31; Molloy *et al.*, *ibid.*; Hallenberger *et al.*, 1992, Nature 360: 358-361), but these substances are toxic *in vivo*. Given the importance of this endoprotease in activation of bacterial toxins, viral structural proteins and bioactive molecules, there is a need for the development of safe and specific furin inhibitors.

SUMMARY OF THE INVENTION

This invention provides safe, specific and effective inhibitors of furin endoprotease that are novel variants of the naturally-occurring protease inhibitor, α_1-antitrypsin (Heeb *et al.*, 1990, J. Biol. Chem 265: 2365-2369; Schapira *et al.*, 1987, J. Clin. Invest. 80: 582-585) and peptides derived therefrom. Use of these novel variants of α_1-antitrypsin is advantageous because α_1-antitrypsin and variants are secreted proteins that are processed by the exocytotic secretory pathway through the Golgi, so synthesis of these proteins in a cell would result in delivery of the inhibitor to the site of furin activity *in vivo*.

In a first embodiment, the invention provides a furin endoprotease inhibitor comprising an α_1-antitrypsin variant protein having an amino acid

sequence comprising the amino acids of the native α_1-antitrypsin molecule (disclosed in Long *et al.*, 1984, Biochemistry 23: 4828-4837, incorporated by reference), except that the sequence at position 355-358 of the native protein (-Ala-Ile-Pro-Met-) is changed to the novel sequence -Arg-X-X-Arg-, wherein each X is any amino acid, at positions 355-358 of the native α_1-antitrypsin amino acid sequence. In a preferred embodiment, the α_1-antitrypsin variant is α_1-antitrypsin Portland and the amino acid sequence at positions 355-358 of the α_1-antitrypsin amino acid Portland sequence is -Arg-Ile-Pro-Arg-.

In a second embodiment, the invention provides a nucleic acid having a nucleotide sequence that encodes an α_1-antitrypsin variant protein having an amino acid sequence comprising the amino acids of the native α_1-antitrypsin molecule, except that the sequence at position 355-358 of the native protein (-Ala-Ile-Pro-Met-) is changed to the novel sequence -Arg-X-X-Arg-, wherein each X is any amino acid, at positions 355-358 in the variant α_1-antitrypsin amino acid sequence. In a preferred embodiment, the α_1-antitrypsin variant is α_1-antitrypsin Portland and the amino acid sequence is -Arg-Ile-Pro-Arg-.

In a third embodiment, the invention provides a recombinant expression construct comprising a nucleic acid having a nucleotide sequence encoding an α_1-antitrypsin variant protein with an amino acid sequence comprising the amino acids of the native α_1-antitrypsin molecule, except that the sequence at position 355-358 of the native protein (-Ala-Ile-Pro-Met-) is changed to the novel sequence -Arg-X-X-Arg-, wherein each X is any amino acid, at positions 355-358 of the variant α_1-antitrypsin amino acid sequence. In a preferred embodiment, the α_1-antitrypsin variant is α_1-antitrypsin Portland and the amino acid sequence is -Arg-Ile-Pro-Arg-. The recombinant expression construct provided by the invention is capable of expressing α_1-antitrypsin variant proteins of the invention in a culture of transformed cells. In a preferred embodiment, the recombinant expression construct comprises a vaccinia virus-based construct. In a more preferred embodiment, the recombinant expression construct comprises a recombinant vaccinia virus vector covalently linked to the nucleic acid encoding the α_1-antitrypsin variant, preferably α_1-antitrypsin Portland.

The invention also provides a cell culture transformed with the recombinant expression construct encoding an α_1-antitrypsin variant capable of expressing the α_1-antitrypsin variant. In a preferred embodiment, the α_1-antitrypsin variant is α_1-antitrypsin Portland. Preferred embodiments of such cell cultures are cultures of bacterial cells, yeast cells, insect cells or mammalian cells.

In another embodiment, the invention provides a homogenous composition of matter comprising an α_1-antitrypsin variant produced by the cell culture according to the teachings of the invention. In a preferred embodiment, the α_1-antitrypsin variant is α_1-antitrypsin Portland.

The invention also provides a furin endoprotease inhibitor comprising α_1-antitrypsin variants capable of blocking endoproteolytic activation of bacterial toxins. In a preferred embodiment, the α_1-antitrypsin variant is α_1-antitrypsin Portland. Pharmaceutically acceptable compositions of the α_1-antitrypsin variants of the invention are also provided comprising a therapeutically effective amount of α_1-antitrypsin variant and a pharmaceutically acceptable carrier or diluent.

Also provided by the invention are peptides having an amino acid sequence of about 4 to about 100 amino acids in length, comprising the amino acid sequence -Arg-X-X-Arg-, wherein each X is any amino acid. In a preferred embodiment, the amino acid sequence is -Arg-Ile-Pro-Arg-.

The invention provides a method of inhibiting bacterial infection of human cells comprising contacting such cells with an α_1-antitrypsin variant of the invention. In a preferred embodiment, the α_1-antitrypsin variant is α_1-antitrypsin Portland. In a preferred embodiment, the bacterial infection is caused by *Corynebacterium diptheriae*. In another preferred embodiment, the bacterial infection is caused by *Bacillus anthracis*. In yet another preferred embodiment, the bacterial infection is caused by *Pseudomonas aeruginosa*.

The invention also provides a method of inhibiting bacterial infection in a human comprising administering a therapeutically effective amount of an α_1-antitrypsin variant of the invention in a pharmaceutically acceptable carrier. In

a preferred embodiment, the α_1-antitrypsin variant is α_1-antitrypsin Portland. In a preferred embodiment, the bacterial infection is caused by *Corynebacterium diptheriae*. In another preferred embodiment, the bacterial infection is caused by *Bacillus anthracis*. In yet another preferred embodiment, the bacterial infection is caused by *Pseudomonas aeruginosa*.

The invention provides a method of treating humans with a bacterial infection comprising administering a therapeutically effective amount of an α_1-antitrypsin variant of the invention in a pharmaceutically acceptable carrier. In a preferred embodiment, the α_1-antitrypsin variant is α_1-antitrypsin Portland. In a preferred embodiment, the bacterial infection is caused by *Corynebacterium diptheriae*. In another preferred embodiment, the bacterial infection is caused by *Bacillus anthracis*. In yet another preferred embodiment, the bacterial infection is caused by *Pseudomonas aeruginosa*.

Another method provided by the invention for treating humans with a bacterial infection comprises administering a combination of a therapeutically effective amount of an α_1-antitrypsin variant and a therapeutically effective amount of a second antibacterial compound in a pharmaceutically acceptable carrier. In a preferred embodiment, the α_1-antitrypsin variant is α_1-antitrypsin Portland. In a preferred embodiment, the bacterial infection is caused by *Corynebacterium diptheriae*. In another preferred embodiment, the bacterial infection is caused by *Bacillus anthracis*. In yet another preferred embodiment, the bacterial infection is caused by *Pseudomonas aeruginosa*.

Pharmaceutically acceptable compositions effective according to the methods of the invention, comprising a therapeutically effective amount of a furin endoprotease inhibitor capable of blocking endoproteolytic activation of bacterial toxins and a pharmaceutically acceptable carrier or diluent, are also provided.

The invention provides a method of inhibiting viral infection of human cells comprising contacting such cells with an α_1-antitrypsin variant according to the invention. In a preferred embodiment, the invention provides a gene therapy delivery system for a nucleic acid encoding an α_1-antitrypsin variant

comprising the recombinant expression construct of the invention and genetic means for delivery and expression of the recombinant expression construct into the cells of an animal. A preferred α_1-antitrypsin variant is α_1-antitrypsin Portland. Pharmaceutically acceptable compositions comprising a therapeutically effective amount of the gene therapy delivery system and a pharmaceutically acceptable carrier or diluent. In a preferred embodiment, the viral infection is caused by Human Immunodeficiency Virus 1 (HIV-1). In another preferred embodiment, the human cells are hematopoietic cells, most preferably T lymphocytes. Other preferred embodiments of viral infections include infection by influenza virus.

The invention also provides a method for inhibiting viral infection in an animal, most preferably a human, comprising administering a therapeutically effective amount of the gene therapy delivery system of the invention in a pharmaceutically acceptable carrier. In a preferred embodiment, the virus is Human Immunodeficiency Virus 1 (HIV-1). In another preferred embodiment, the virus is cytomegalovirus.

The invention provides a method of treating humans infected with a virus comprising administering a therapeutically effective amount of the gene therapy delivery system of the invention in a pharmaceutically acceptable carrier. In a preferred embodiment, the virus is Human Immunodeficiency Virus 1 (HIV-1). In another preferred embodiment, the virus is influenza virus.

The invention provides a method of treating humans infected with a virus comprising administering a combination of a therapeutically effective amount of the gene therapy delivery system of the invention and a therapeutically effective amount of a second antiviral compound in a pharmaceutically acceptable carrier. In a preferred embodiment, the virus is Human Immunodeficiency Virus 1 (HIV-1) and the second antiviral compound is azidothymidine. In another preferred embodiment, the virus is influenza virus.

The invention also provides a method for treating virus-associated immunosuppression in a human comprising administering a therapeutically effective amount of the gene therapy delivery system of the invention in a

pharmaceutically acceptable carrier. In a preferred embodiment, the virus is Human Immunodeficiency Virus 1 (HIV-1).

Pharmaceutically acceptable compositions effective according to the methods of the invention, comprising a therapeutically effective amount of the gene therapy delivery system encoding α_1-antitrypsin variants having antiviral properties and a pharmaceutically acceptable carrier or diluent, are also provided. In a preferred embodiment, the α_1-antitrypsin variant is α_1-antitrypsin Portland

The invention also provides a method of inhibiting proteolytic processing of a biologically active protein or peptide in a cell comprising contacting such cells with the gene therapy delivery system of the invention. Preferred biologically active proteins are pro-β-nerve growth factor, blood coagulation factor protein Factor IX, pro-von Willibrand factor, complement factor C3 and renin.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 illustrates production of native α_1-antitrypsin (Lane 1), α_1-antitrypsin Pittsburgh (Lane 2) and α_1-antitrypsin Portland (Lane 3) by BSC-40 cells infected with vaccinia virus recombinant constructs.

Figure 2 shows inhibition of thrombin and furin by native α_1-antitrypsin (striped bars), α_1-antitrypsin Pittsburgh (dotted bars) and α_1-antitrypsin Portland (stipled bars) *in vitro*.

Figure 3 is an SDS-PAGE analysis of inhibition showing proteolytic processing of pro-β-NGF by α_1-antitrypsin and variants.

Figure 4 depicts Western blot analysis showing inhibition of proteolytic processing of HIV gp160 by α_1-antitrypsin and variants.

Figure 5 illustrates the experimental protocol for assaying the gp41-mediated fusogenic capacity of gp160-producing IP/IR erythroleukemia cells to form syncytia with $CD4^+$ human HeLa cells in the presence and absence of co-expressed α_1-antitrypsin and variants.

Figure 6 illustrates the results of the syncytium experiments described in

Example 5.

Figure 7 shows an autoradiograph of α_1-antitrypsin Portland-mediated inhibition of pro-β-NGF proteolytic processing in VV:mNGF-infected BSC-40 cells (Panel A) and quantitation of these results as a percentage of the autoradiographic density of the processed band (β-NGF) relative to the total autoradiographic density (the sum of the β-NGF band plus the pro-β-NGF band).

Figure 8 depicts the structure of recombinant expression constructs pRep4ΔL/Rpα_1-PIT and pRep4ΔL/Rpα_1-PDX.

Figure 9 illustrates HIV-infected cultures of HeLa CD4$^+$ clone 1022 cells (Panel B) and two independent transfectants, PDX-4 (Panel C) and PDX-6 (Panel A) four days post-infection.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The production of proteins such as the α_1-antitrypsin Portland from cloned genes by genetic engineering is well known. *See, e.g.*, U.S. Patent No. 4,761,371 to Bell *et al.* at Col. 6 line 3 to Col. 9 line 65. (The disclosure of all U.S. patent references cited herein is to be incorporated herein by reference.) The discussion which follows is accordingly intended as an overview of this field, and is not intended to reflect the full state of the art. For the purposes of this discussion, the α_1-antitrypsin variant α_1-antitrypsin Portland will be used as an exemplar; it will be understood that the discussion applies to and the invention encompasses all the α_1-antitrypsin variants of the invention.

DNA which encodes α_1-antitrypsin Portland may be obtained, in view of the instant disclosure, by chemical synthesis, or by *in vitro* mutagenesis as described in Example 2 herein of the native α_1-antitrypsin DNA sequence. Such native α_1-antitrypsin DNA can be obtained by screening reverse transcripts of mRNA from appropriate cells or cultured cell lines, by screening genomic libraries from appropriate cells, or by combinations of these procedures. Screening of mRNA or genomic DNA may be carried out with probes generated from the known α_1-antitrypsin gene sequence. Probes may be labeled with a detectable group such as a fluorescent group, a radioactive atom or a

chemiluminescent group in accordance with know procedures and used in conventional hybridization assays (*see* Sambrook *et al.*, 1990, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, N.Y.). In the alternative, α_1-antitrypsin gene sequences may be obtained for *in vitro* mutagenesis by use of the polymerase chain reaction (PCR) procedure, with the PCR oligonucleotide primers being produced from the native α_1-antitrypsin gene sequence. *See* U.S. Patent Nos. 4,683,195 to Mullis *et al.* and 4,683,202 to Mullis.

α_1-antitrypsin variants such as α_1-antitrypsin Portland may be synthesized in host cells transformed with a recombinant expression construct comprising a DNA sequence encoding an α_1-antitrypsin variant. Such a recombinant expression construct can also be comprised of a vector that is a replicable DNA construct. Vectors are used herein either to amplify DNA encoding α_1-antitrypsin Portland and/or to express DNA encoding α_1-antitrypsin Portland. For the purposes of this invention, a recombinant expression construct is a replicable DNA construct in which a DNA sequence encoding an α_1-antitrypsin variant is operably linked to suitable control sequences capable of effecting the expression of the α_1-antitrypsin variant in a suitable host. The need for such control sequences will vary depending upon the host selected and the transformation method chosen. Generally, control sequences include a transcriptional promoter, an optional operator sequence (in bacteria) or enhancer sequence (in eukaryotic cells) to control transcription, a sequence encoding suitable mRNA ribosomal binding sites, and sequences which control the termination of transcription and translation. Amplification vectors do not require expression control domains. All that is needed is the ability to replicate in a host, usually conferred by an origin of replication, and a selection gene to facilitate recognition of transformants.

DNA regions are operably linked when they are functionally related to each other. For example: a promoter is operably linked to a coding sequence if it controls the transcription of the sequence; a ribosome binding site is operably linked to a coding sequence if it is positioned so as to permit

translation. Generally, operably linked means contiguous and, in the case of leaders sequences, contiguous and in the same translational reading frame.

Vectors useful for practicing the present invention include plasmids, viruses (including phage), retroviruses, and integratable DNA fragments (i.e., fragments integratable into the host genome by homologous recombination). The vector may replicate and function independently of the host genome, or may, in some instances, integrate into the genome itself. Suitable vectors will contain replicon and control sequences which are derived from species compatible with the intended expression host. A preferred vector is the plasmid pZV*neo*, useful for producing recombinant expression constructs based on homologous recombination with vaccinia virus sequences.

A preferred embodiment of the recombinant expression constructs of this invention comprise vaccinia virus sequences capable of infecting mammalian cells and expressing α_1-antitrypsin variant, as described below in Example 2.

Transformed host cells are cells which have been transformed or transfected with a recombinant expression construct made using recombinant DNA techniques and comprising sequences encoding an α_1-antitrypsin variant. Transformed host cells may express α_1-antitrypsin Portland, but host cells transformed for purposes of cloning or amplifying DNA need not express these sequences.

Cultures of cells, including cells derived from multicellular organisms, are a desirable hosts for recombinant α_1-antitrypsin Portland synthesis. In principal, any cell culture is useful that is capable of being transformed with an appropriate recombinant expression construct and expressing α_1-antitrypsin protein. The invention is preferably practiced with bacterial, yeast, insect or mammalian cells, however, mammalian cells are more preferred, as illustrated in the Examples. Propagation of bacteria and yeast is well known in the art, and propagation of mammalian cells in cell culture has become a routine procedure. *See* Tissue Culture, Academic Press, Kruse & Patterson, editors (1973). Most preferred mammalian cells are BSC-40 African green monkey kidney cells, but other cells, such as human 293 cells, VERO and HeLa cells, Chinese hamster

ovary (CHO) cell lines, and WI138, BHK, COS-7, CV, and MDCK cells, are also useful.

The invention provides homogeneous compositions of α_1-antitrypsin Portland produced by transformed cells as provided herein. Such homogeneous compositions are intended to be comprised of mammalian α_1-antitrypsin Portland protein that comprises at least 90% of the protein in such homogenous composition.

The recombinant expression constructs of the present invention are useful in molecular biology to transform cells *in vivo* in an animal as a method for protecting the animal from viral or other infection in cells that express furin or a furin-like endoprotease activity that can be inhibited by α_1-antitrypsin Portland. The invention provides a gene therapy delivery system comprising the recombinant expression constructs of the invention in a configuration that enables safe and efficient introduction of these sequences into appropriate cells and expression of α_1-antitrypsin Portland. For such purposes, retroviral vectors as described in U.S. Patent No. 4,650,764 to Temin & Watanabe or U.S. Patent No. 4,861,719 to Miller may be employed, for example. The recombinant expression constructs of the invention may also be used in gene therapy carried out homologous recombination. *See generally* Thomas & Capecchi, 1987, Cell 51: 503-512; Bertling, 1987, Bioscience Reports 7: 107-112; Smithies *et al.*, 1985, Nature 317: 230-234. Additionally, alteration of endogenous α_1-antitrypsin sequences to produce α_1-antitrypsin Portland in cells carrying such altered α_1-antitrypsin sequences can also be achieved using homologous recombination or other techniques. Transgenic animals, the tissues of which express the α_1-antitrypsin Portland variant, are also envisioned as additional objects of this invention.

The peptides of this invention may be generated and/or isolated by any means known in the art. It is within the skill of those of ordinary skill in the art to isolate or chemically synthesize a nucleic acid encoding each of the peptides of the invention. Such nucleic acids are advantageously utilized as components of recombinant expression constructs, wherein the nucleic acids are

operably linked with transcriptional and/or translational control elements, whereby the recombinant expression constructs of the invention are capable of expressing the peptides of the invention in cultures of cells, preferably eukaryotic cells, most preferably mammalian cells, transformed with such recombinant expression constructs.

The peptides of the invention may be advantageously synthesized by any of the chemical synthesis techniques known in the art, particularly solid-phase synthesis techniques, for example, using commercially-available automated peptide synthesizers. Such peptides may be provided as linear peptides encompassing the amino acid sequence -Arg-X-X-Arg-, where each X is any aminio acid. These peptides may also be provided in the form of combination peptides, wherein the peptides comprising the combination are linked in a linear fashion one to another, with or without separation by "spacer" amino acids allowing for selected conformational presentation. Also provided are branched-chain combinations, wherein the component peptides are covalently linked *via* functionalities in amino acid sidechains of the amino acids comprising the peptides.

The invention also provides antibacterial and antiviral methods. The invention provides methods for blocking endoproteolytic activation of bacterial toxins. Bacterial targets of the antibacterial methods provided by this invention include but are not limited to any bacteria that produces an endoproteolytically-activated toxin, such as diptheria toxin produced by *Corynebacterium diptheriae*, exotoxin A of *Pseudomonas aurigenosa*, tetanus toxin, the enterotoxins of *Escherichia coli* and *Vibrio cholerae*, protective antigen of *Bacillus anthracis* and the neurotoxin and C2 toxin of *Clostridium botulinum*. Preferred toxins are those that are proteolytically processed at a consensus furin recognition site (-Arg-Xaa-Xaa-Arg↓-). Preferred embodiments include *Corynebacterium diptheriae*, *Pseudomonas aeruginosa* and *Bacillus anthracis*.

Viral targets of antiviral methods provided include but are not limited to picornaviruses (*e.g.*, poliovirus and rhinovirus); orthomyxoviruses (*e.g.*, influenza virus); paramyxoviruses (*e.g.*, measles virus and mumps virus);

coronaviruses; rhabdoviruses (*e.g.*, rabies virus and vesicular stomatitis virus); togaviruses (*e.g.*, Semliki Forest virus and yellow fever virus); bunyaviruses (*e.g.*, California encephalitis virus); arenaviruses (*e.g.*, Lassa fever virus); rubella virus; reoviruses (*e.g.*, Colorado tick fever virus); hepatitis viruses; adenoviruses; herpesviruses (*e.g.*, herpes simplex virus); and oncogenic viruses, including papilloma viruses, RNA tumor viruses, or retroviruses, and lentiviruses (*e.g.*, human immune deficiency virus). The most preferred viruses are the human immunodeficiency viruses (HIV-1 and HIV-2).

Cells intended to be protected by the methods provided by this invention include but are not limited to human, canine, bovine, murine, leporine, porcine, ovine, simian, feline, hircine, and equine cells. The preferred cells are human cells. More preferred cells are human T lymphocytes (T cells), and the most preferred human T cells are those human T cells expressing the cell surface antigen CD4.

The methods of the present invention may be used to treat donated human blood or plasma to protect transfusion recipients from viral infection from contaminating virus. The methods of the present invention may be used to treat human semen to protect embryos derived from such semen, and mothers bearing such embryos or impregnated with such semen, from contaminating virus. In a preferred embodiment, the contaminating virus is HIV-1.

The present invention provides methods for inhibiting viral infection in a human. The invention also provides for treating a human infected with a virus. Another embodiment of the present invention includes methods for treating immunosuppression in a human associated with viral infection. Yet another embodiment of the present invention provides a method of prophylaxis for treating a human exposed to infection with a virus, in particular those directly at risk of infection as a result of intimate contact with humans infected with a virus of tissues or bodily fluids contaminated by a virus. The preferred virus of these embodiments of the invention is HIV-1. The invention provides pharmaceutically acceptable compositions effective for use with the methods provided by the invention comprising the peptides of the invention and a

pharmaceutically acceptable carrier.

The invention also provides methods for inhibiting proteolytic processing of a biologically active protein or peptide in a cell comprising contacting such cells with the gene therapy delivery system of the invention. The methods of the invention encompass inhibition of proteolytic processing of any biologically active molecule that is proteolytically processed by furin *in vivo* or *in vitro*, including but not limited to peptide hormones, neuropeptides, growth factors, coagulation factors, serum albumin, cell surface receptors, and adhesion molecules. Preferred biologically active proteins are pro-β-nerve growth factor, blood coagulation factor protein Factor IX, pro-von Willibrand factor, complement factor C3 and renin, for alleviation of pathological conditions and disease states in an animal, preferably a human, associated with over-expression, over-production or otherwise inappropriate synthesis of such biologically-active proteins.

Preparation of pharmaceutically acceptable compositions provided by the present invention can be prepared using methods well known to those with skill in the art. Any of the common carriers such as sterile saline solution, plasma, etc., can be utilized for preparing the pharmaceutical compositions provided by the invention. Routes of administration include but are not limited to oral (including inhalation into the lungs), intravenous, parenteral, rectal, optical, aural and transdermal. The pharmaceutical compositions of the invention may be administered intravenously in any conventional medium for intravenous injection such as an aqueous saline medium, or in blood plasma medium. Such medium may also contain conventional pharmaceutical adjunct materials such as, for example, pharmaceutically acceptable salts to adjust the osmotic pressure, buffers, preservatives and the like. Among the preferred media are normal saline and plasma.

The following Examples illustrate certain aspects of the above-described method and advantageous results (also disclosed in Anderson *et al.*, 1993, J. Biol. Chem. 268: 24887-24891). The following Examples are shown by way of illustration and not by way of limitation.

EXAMPLE 1

Production of Furin Endoprotease in African Green Monkey Cells *In Vitro*

Human furin was synthesized for inhibition experiments as described in Bresnahan *et al.* (1990, J. Cell Biol. 111: 2851-2859) and Molloy *et al.* (1992, J. Biol. Chem. 267: 16396-14402; both hereby incorporated by reference). Briefly, a furin cDNA (van den Ouweland *et al.*, 1992, Nucleic Acids Res. 18: 664) encoding a truncated but functional furin protein was inserted into the multiple cloning site of a vaccinia virus vector (*see* Hruby *et al.*, 1986, Meth. Enzymol. 124: 295-309) and used to infect BSC-40 African green monkey kidney cells (ATCC Accession No. CCL 26, American Type Culture Collection, Rockville, MD). The cells were incubated in serum-free defined media for 24 hours and then harvested at 4°C and disrupted by 20-30 strokes in a Dounce homogenizer (Kontes Glass Co., Vineland, OH). The lysate was cleared by low-speed centrifugation (1000*g*, 5 min) and the supernatant then subjected to ultracentrifugation at 100,000*g* for 1 hr. The pellet was resuspended in 200μL of a buffer comprising 10mM HEPES [*N*-(2-hydroxyethyl)piperazine-*N'*-(2-ethanesulfonic acid), Sigma Chemical Co., St. Louis, MO], pH 7.5 and stored at 4°C until use.

Alternatively, a soluble, truncated derivative of furin lacking the carboxyl-terminal 81 amino acids was used (*see* Molloy *et al.*, *ibid.*, incorporated by reference, for a more detailed description of this molecule). Briefly, BSC-40 cells were infected with a vaccinia virus vector containing the truncated furin cDNA, grown for 16-18 h in serum-free defined media and then harvested. The cells were disrupted and subjected sequentially to centrifugation at 1000*g* and 10,000*g* at 4°C. The supernatant was passed through a 0.2μm filter and then diluted approximately 1:1 with a buffer consisting of 20mM BisTris (pH 7.0) and 1mM 2-mercaptoethanol. This mixture was then applied to a Mono Q HR 5/5 fast protein liquid chromatography anion exchange column (Pharmacia LKB Biotechnology Inc.) and the truncated furin derivative eluted with a gradient of 0-750mM sodium chloride and stored at 4°C until use.

EXAMPLE 2

In Vitro Mutagenesis and Production of Native and Variant Species of α_1-Antitrypsin

A novel α_1-antitrypsin variant, termed α_1-antitrypsin Portland, was produced by *in vitro* mutagenesis of a cloned cDNA encoding the naturally-occurring variant α_1-antitrypsin Pittsburgh (Lewis *et al.*, 1978, Blood 51: 129-137; Owen *et al.*, 1983, N. Engl. J. Med. 309: 694-698). A full length cDNA encoding α_1-antitrypsin Pittsburgh (which is identical to the sequence disclosed in Long *et al.*, *supra*, except for a $Met_{358} \rightarrow Arg_{358}$ mutation) was subcloned into M13mp19 phage using standard techniques (*see* Sambrook *et al.*, *ibid.*, Chapter 15). The following mutagenesis primer was then annealed to the sequence of single-stranded phage DNA corresponding to codons 352-358 of the α_1-antitrypsin Pittsburgh sequence:

5'-TTTTTAGAGCGCATACCCAG-3'
(SEQ ID No:1)

The underlined sequence encodes the mutagenized codon. After annealing, the mutagenesis primer was extended using the Klenow fragment of *E. coli* DNA polymerase in the presence of the four deoxynucleotide triphosphates. Clones having the mutagenized sequence were then grown and selected in *E. coli*, and DNA sequencing of the appropriate portion of the sequence of selected mutagenized clones was performed to confirm successful mutagenesis. The mutagenized α_1-antitrypsin cDNA sequences were then subcloned into the vaccinia virus recombination vector pZV*neo* and used to produce vaccinia virus recombinants as described in Example 1 (and described in more detail in Hayflick *et al.*, 1992, J. Neurosci. 12: 705-717, hereby incorporated by reference).

As a result of mutagenesis, the sequence of α_1-antitrypsin Pittsburgh (Ala_{355}-Ile-Pro-Arg_{358}) was changed to the novel sequence of α_1-antitrypsin Portland (Arg_{355}-Ile-Pro-Arg_{358}). Vaccinia virus constructs containing native α_1-antitrypsin (VV:α_1-NAT; Ala_{355}-Ile-Pro-Met_{358}), α_1-antitrypsin Pittsburgh (VV:α_1-PIT) and α_1-antitrypsin Portland (VV:α_1-PDX) were each used to infect

BSC-40 cells. Such infected cells secrete each of the α_1-antitrypsins into the culture media, and native α_1-antitrypsin and variants were isolated from culture media from appropriately-infected BSC-40 cells by passage of such media over a Mono Q HR 5/5 high pressure liquid chromatography anion exchange column (Pharmacia LKB Biotechnology Ltd., Stockholm, Sweden) and eluted using a linear gradient (0.05 -> 0.5M) of sodium chloride in 50mM Tris-HCl (pH 8.0), as described further in Molloy *et al.* Production of native α_1-antitrypsin (Lane 1) and variants Pittsburgh (Lane 2) and Portland (Lane 3) was confirmed by Western blot hybridization (*see* Sambrook *et al.*, *ibid.*, Chapter 18) as shown in Figure 1.

EXAMPLE 3

In Vitro Characterization of Furin Endoprotease Inhibition by α_1-Antitrypsin Portland

α_1-antitrypsin and variants Pittsburgh and Portland were assayed for the ability to inhibit furin endoprotease *in vitro* essentially as described in Molloy *et al.* (*ibid.*). Briefly, 25μL of the resuspended furin preparation described in Example 1 was incubated with each of the α_1-antitrypsins (at a final concentration of 10μg/mL) for 20 min at 37°C in a buffer comprising 100mM HEPES (pH 7.5), 1mM $CaCl_2$, 1mM 2-mercaptoethanol and 0.5% Triton-X 100. Substrate [(*N*-butoxycarbonyl)-Arg-Val-Arg-Arg-(4-methylcoumaryl-7-amide); Peninsula Laboratories, Belmont, CA] was then added to a final concentration of 50 μM and incubated for 30 min at 37°C. The amount of liberated aminomethylcoumarin was then determined by fluorimetry (excitation wavelength = 380nm; emission wavelength = 460nm) using a spectrofluorimeter (Perkin Elmer, Waterbury, CT, Model LS3). The results of this experiment are shown in Figure 2. For comparison, each of the α_1-antitrypsins were also incubated with thrombin and assayed for thrombin peptidase activity colorometrically using *benzoyl*-Phe-Val-Arg-(*para*-nitroanilide) as substrate. These results are also shown in Figure 2. Native α_1-antitrypsin was unable to inhibit either furin or thrombin endoprotease activity (striped

29.10.94

TABLE I

Enzyme	Conc.[a]	α_1-NAT[b]	α_1-PIT[b]	α_1-PDX[b]
Elastase	100	13	98	101
Thrombin	100	94	8	95
Furin	100	101	97	0.04

a = relative concentration of each serpin inhibitor
b = percent endoprotease activity in presence of each serpin

bars). α_1-antitrypsin Pittsburgh specifically inhibited thrombin but not furin (dotted bars). The novel variant α_1-antitrypsin Portland (stipled bars) had no effect on thrombin endoprotease activity, but specifically and essentially quantitatively inhibited furin endoprotease activity. Table I provides the results of a comparison between the levels of inhibition of elastase [assayed colorometrically with (*N*-butoxycarbonyl)-Ala-Ala-Pro-Ala-(*para*-nitroanilide)as substrate]. Elastase activity was specifically inhibited by native α_1-antitrypsin (α_1-NAT), thrombin was specifically inhibited by α_1-antitrypsin Pittsburgh (α_1-PIT), and furin was specifically inhibited by α_1-antitrypsin Portland (α_1-PDX). α_1-PDX was found to be greater than 3000-fold more effective than α_1-PIT in inhibiting furin, having a $K_{\frac{1}{2}}$ of 0.03μg/mL, equivalent to 0.4nM). α_1-PDX is also greatly attenuated in thrombin inhibitory activity (>300-fold) compared with α_1-PIT.These results demonstrated that the novel α_1-antitrypsin structural variant (α_1-PDX) disclosed herein has novel functional properties that make it useful as a specific furin endoprotease inhibitor.

EXAMPLE 4

In Vivo Characterization of Furin Endoprotease Inhibition by α_1-Antitrypsin Portland

α_1-antitrypsin Portland was assayed for the ability to inhibit furin endoprotease *in vivo*. BSC-40 cells were co-infected with a vaccinia virus vector encoding the α_1-antitrypsin Portland as described in Example 2 and a vaccinia virus vector encoding pro-β-nerve growth factor (β-NGF), a neuropeptide growth factor known to be processed by furin at the consensus furin site -Arg-Ser-Lys-Arg↓- (Bresnahan *et al.*, *ibid.*) and secreted into the cell growth media. Co-infected cells were incubated in the presence of [^{35}S]-methionine for 4 h after infection and the cell media harvested. Media samples were then immunoprecipitated with NGF-specific antibodies and assayed by sodium dodecyl sulfate/ polyacrylamide gel electrophoresis (SDS-PAGE) and visualized by fluorography using standard techniques (as described in Sambrook *et al.*, *ibid.*).

The results of these experiments are shown in Figure 3. Cells infected with wild-type vaccinia virus secreted no detectable β-NGF into the culture media (Lane A), whereas cells infected with the vaccinia virus vector encoding β-NGF secreted both processed (13 kilodaltons, kD) and unprocessed (≈35 kD) forms of β-NGF; co-infection of such cells with wild-type vaccinia virus had no effect on this pattern of β-NGF production (Lane C). Similarly, BSC-40 cells co-infected with the β-NGF construct and with vaccinia virus constructs encoding native α_1-antitrypsin (Lane D) and α_1-antitrypsin Pittsburgh (Lane E) also produced both the processed and unprocessed forms of β-NGF. In contrast, cells co-infected with the β-NGF construct and with vaccinia virus constructs encoding α_1-antitrypsin Portland (Lane F) produced only the unprocessed form of β-NGF, demonstrating that α_1-antitrypsin Portland is capable of inhibiting furin-mediated endoprotease processing of bioactive pro-peptides *in vivo*.

EXAMPLE 5

Inhibition of Furin-Mediated Processing of Human Immunodeficiency Virus gp160 by α_1-Antitrypsin Portland

The experiments described in Example 4 were repeated using a vaccinia virus construct encoding the Human Immunodeficiency virus (HIV-1) glycoprotein gp160. This precursor protein is known to be proteolytically processed into two membrane-associated proteins: gp120 (which binds the HIV receptor CD4 on the cell surface of target host cells) and gp41 (which provides a fusogenic activity that mediates viral entry into the cell) *in vivo*. Proteolytic processing at the furin consensus site -Arg-Glu-Lys-Arg↓- is a necessary step in maturation and release of HIV viral particles. Cell membranes from cells infected with vaccinia virus constructs were isolated as described in Example 1. Proteins from such membrane preparations were resolved by SDS-PAGE and specifically identified by Western blot analysis (*see* Sambrook *et al.*, *ibid.*, Chapter 18) using antibodies against HIV proteins (*see* Anderson *et al.*, *supra*).

The results of this experiment are shown in Figure 4. Cells infected with wild-type vaccinia virus produced no detectable HIV-related peptides (Lane A).

In cells infected with the vaccinia virus vector encoding gp160, both the unprocessed protein and processing products, gp120 and gp41 were produced. BSC-40 cells co-infected with the gp160 construct and with vaccinia virus constructs encoding native α_1-antitrypsin (Lane C) and α_1-antitrypsin Pittsburgh (Lane D) also produced both unprocessed gp160 and processed gp120 and gp41. Cells co-infected with the gp160 construct and with vaccinia virus constructs encoding α_1-antitrypsin Portland (Lane E), on the other hand, produced only unprocessed gp160. These results demonstrated that α_1-antitrypsin Portland is capable of inhibiting furin-mediated endoprotease processing of bioactive viral proteins *in vivo*, and immediately suggested a method for treating viral infection by inhibiting viral protein processing.

In another series of experiments, α_1-antitrypsin Portland-mediated inhibition of proteolytic processing of gp160 was assayed to determine the functional consequences of such inhibition. As described above, processing of gp160 results in the production of gp41, a protein that provides a fusogenic activity important for viral entry into target cells. Expression of gp120 and gp41 on the surface of IP/IR erythroleukemia cells (*see* Spiro *et al.*, 1988, J. Virol. 63: 4434-4437) promotes cell fusion and syncytium formation with cells expressing the gp120 target, CD4, at the cell surface. In these experiments, IP/IR cells were co-infected with vaccinia virus expression constructs encoding gp160 and each of the α_1-antitrypsins to determine the effect of inhibition of gp160 processing on the fusogenic capacity of the cells. This experimental protocol is shown in Figure 5. Briefly, IP/IR cells were co-infected with the vaccinia virus gp160 construct, either alone or co-infected with each of the α_1-antitrypsin constructs described in Example 2. The cells were incubated for 8-16 hours, collected and then overlaid onto a monolayer of $CD4^+$ human HeLa cells [a modification of parental HeLa cells (ATCC No. CCL 2) that express the CD4 cell surface protein, as described in Kabat *et al.*, 1994, J. Virol. __: ____-____). These cells were incubated for an additional 8-12 hours, and syncytium formation detected by staining with crystal violet and observed by phase-contract microscopy.

The results of these experiments are shown in Figure 6. Panel A shows the results of HeLa/CD4$^+$ cell overlay with IP/IR cells infected with vaccinia virus recombinants encoding HIV gp160, and Panel B shows results of HeLa/CD4$^+$ cell overlay experiments using IP/IR cells infected with HIV gp160 vaccinia recombinants co-infected with wild type vaccinia virus. Syncytia formation (and hence proper proteolytic processing of gp160 to gp120 and gp41) in the cells of each of these infected co-cultures was evidenced by the large number of multinucleated cells in each of the cultures. Panel C shows the results of HeLa/CD4$^+$ cell overlay experiments using IP/IR cells infected with wild-type vaccinia virus; essentially no multinucleated cells were seen in such cultures. Panels D and E show the results of HeLa CD4$^+$ cell overlay experiments using IP/IR cells co-infected with vaccinia virus recombinants encoding gp160 and native (Panel C) and the Pittsburgh variant (Panel D) of α_1-antitrypsin. Co-expression of native or variant Pittsburgh α_1-antitrypsin had no effect on syncytia formation caused by gp41. Panel F shows the effect on HIV gp41-mediated syncytia formation of HeLa CD4$^+$ cell overlay experiments using IP/IR cells co-infected with vaccinia virus recombinants encoding gp160 and α_1-antitrypsin Portland in HeLa/CD4$^+$ cells. Syncytia formation is completely abolished in these cultures, and the cells of such cultures looked identical to cells in overlay experiments using IP/IR cells infected with wild-type vaccinia virus as seen in Panel C. These results demonstrate that inhibition of gp160 processing by α_1-antitrypsin Portland eliminates the fusogenic activity of viral gp41 and suggests that such inhibition may provide a method for treating HIV infection *in vivo* and *in vitro*.

EXAMPLE 6

Inhibition of Furin-Mediated Processing of β-Nerve Growth Factor by Direct Application of α_1-Antitrypsin Portland

The ability of α_1-antitrypsin Portland to inhibit proteolytic processing by addition of the inhibitor to the cellular growth media was demonstrated in the following assay. BSC-40 cells were grown to confluence in 35mm tissue culture

plates (Falcon Microbiological Supply Co., Lincoln Park, NJ) and then infected with recombinant vaccinia virus encoding a functional pro-β-NGF protein (VV:mNGF) as described in Example 4. Four hours prior to infection, the cellular growth media was removed and replaced with serum-free defined media (MCDB 202 media, made as described in McKeeton *et al.*, 1977, Dev. Biol. Std. 37: 97-108) supplemented with either 0, 0.5 or 50 μg/mL α_1-antitrypsin Portland (α_1-PDX), prepared as described in Example 2. After this 4 h incubation, the cells were infected with VV:mNGF at a multiplicity of infection of 5 pfu/cell. In one culture, the pre-incubated cells were transfected with VV:mNGF at a m.o.i. of 2 and co-infected with VV:α_1-PDX (*see* Example 2) at a m.o.i. of 5. After virus inoculation, α_1-PDX was added to the culture media of each infected plate at the same concentration as in the pre-incubation period.

Eighteen hours post-infection the media was removed from each plate and the cells metabolically labeled by incubation for 5 h in media containing [^{35}S]-methionine and [^{35}S]-cysteine (500μCi) and α_1-PDX at the appropriate concentration for each plate. After labeling, the culture media from each plate was harvested and NGF peptides immunoprecipitated, resolved by SDS-PAGE and visualized by fluorography as described in Example 4.

The results of this experiment are shown in Figure 7. Panel A is an autoradiograph of SDS-PAGE analysis of immunoprecipitated NGF peptides from each of the infected cultures, in which lane 1 contains the results from the culture incubated with α_1-PDX at 0 μg/mL; lane 2 contains the results from the culture incubated with α_1-PDX at 0.5 μg/mL; lane 3 contains the results from the culture incubated with α_1-PDX at 50 μg/mL; and lane 4 contains the results from the culture co-infected with VV:α_1-PDX. As this autoradiographic evidence shows, incubation of cell cultures with α_1-PDX protein resulted in marked attenuation of furin-directed proteolytic processing of pro-β-NGF in a dose-dependent manner. The autoradiographic data was quantitated by densitometry as shown in Panel B; the percent relative amount of inhibition of proteolytic processing is represented as a percentage of the density of the

processed (β-NGF) band relative to the density of the sum of the processed plus the unprocessed (pro-β-NGF) band. The Figure shows that α_1-PDX at 50 μg/mL reduces the amount of proteolytic processing about five-fold [47% (0 μg/mL) *versus* 10% (50 μg/mL)]. Essentially no processing is seen in the co-infected cell line, consistent with the results described in Example 4 above.

This dose-dependent attenuation of furin-directed proteolytic processing following administration of α_1-PDX protein directly to cells in culture demonstrates the feasibility of a protein-based therapeutic approach directed at inhibiting furin-directed proteolytic maturation of a variety of biologically-important protein molecules, which is additionally advantageous due to the lack of thrombin inhibition exhibited by α_1-PDX protein as disclosed herein.

EXAMPLE 7

Construction of an HIV-LTR-based Recombinant Expression Vector Encoding α_1-Antitrypsin Portland

In order to stably transfect cells with a recombinant expression construct capable of expressing α_1-PDX protein *in vivo*, the following expression vector was made, wherein expression of α_1-PDX was mediated by the long terminal repeat (LTR) sequences of HIV-2. The completed plasmid is shown in Figure 8.

A 470bp fragment was excised from the recombinant plasmid pGEM/RRE by *Sma*I/*Hin*dIII digestion and then blunt-end cloned into the *Bam*HI site of the plasmid pRep4 (Invitrogen, San Diego, CA). Restriction enzyme-generated overhangs were blunt-ended using Klenow polymerase (Pharmacia, Upsala, Sweden) using conventional techniques (*see* Sambrook *et al.*). The 470bp fragment contains the *rev* responsive element of HIV-HXB2, comprising nucleotides 7621-8140 (Malim *et al.*, 1989, Nature 338: 254-257). The Rous Sarcoma Virus 3' LTR was excised from the resulting plasmid by a *Sal*I (partial blunt)/*Hin*dIII digest, and replaced by ligation with an *Eco*RI(blunt)/*Hin*dIII digestion fragment from pBennCAT (NIH AIDS Research Program, Bethesda, MD) containing the HIV LTR (nucleotides -450 to +80).

The resulting expression plasmid was termed pRep4/RRE.

Plasmids capable of expressing α_1-PIT or α_1-PDX cDNAs were constructed by inserting each of the cDNA molecules into pRep4/RRE between the HIV LTR and the RRE sequence in the proper orientation. This was done by excising α_1-PIT cDNA sequences from pA1AT PIT (Long *et al.*, *supra*) using *Eco*RI (blunt) and *Xho*I digestion, or by excising α_1-PDX cDNA sequences from pAIAT PDX using *Sma*I and *Xho*I digestion, and cloning each cDNA into *Hin*dIII (blunt)/*Xha*I-digested pRep4/RRE. In a final step, the Epstein Barr virus origin of replication (ORIP) and nuclear antigen (EBNA-1) sequences were excised from the final versions of each of the α_1-antitrypsin variant expression constructs as follows. pRep4/RRE was digested with *Spe*I and *Cla*I, blunt-ended as described above, and religated to itself (*see* Figure 8). A *Xba*I/*Sac*II fragment from the pRep4/RRE plasmid so modified was then swapped for the corresponding fragment in each of the α_1-antitrypsin variant plasmids described above. This resulted in plasmids having the structure illustrated in Figure 8 and encoding each of the α_1-antitrypsin variants α_1-PIT (plasmid pRep4ΔL/Rpα_1-PIT) and α_1-PDX (plasmid pRep4ΔL/Rpα_1-PDX).

EXAMPLE 8

Establishment of Human CD4$^+$ Cell Lines Expressing α_1-Antitrypsin Portland

The pRep4ΔL/Rpα_1-PDX plasmid contains a functional hygromycin resistance gene (*see* Figure 8) capable of conferring hygromycin resistance to eukaryotic cells (*see* Product Catalog, Invitrogen). Ten micrograms of this plasmid DNA were introduced into HeLa/CD4$^+$ clone 1022 cells (described in Example 5 above) using a modified calcium phosphate precipitation technique (Chen & Okayama, 1988, Molec. Cell. Biol. 8: 123-130, as further described in Sambrook *et al.*, *ibid.*). Two days after transfection the cells were placed on selective media RPMI60/10% fetal calf serum supplemented with 100 μg/mL hygromycin (Sigma Chemical Co.) and maintained in this media throughout the experiment. Cell clones resistant to the drug appeared after about two weeks in

selective media, and individual clones were isolated and expanded for subsequent experiments using conventional techniques [*see* Tissue Culture, Academic Press, Kruse & Patterson, editors (1973)]. Two such clones (termed PDX-4 and PDX-6) were used in the following experiments.

Cell lines PDX-4, PDX-6 and parental HeLa $CD4^+$ clone 1022 were seeded in 6-well plates (Falcon) at a density of 2 x 10^5 cells/plate. Cells cultures were then infected in duplicate with 2mL of media containing HIV strain LAV (NIH AIDS Research Program) having a reverse transcriptase activity of about 70,000 cpm/mL. Cell cultures were examined four days post-infection for cytopathic effects and syncytia formation. Such cultures are shown in Figure 9. The parental HeLa clone 1022 cells (Panel B) exhibited numerous syncytia and cytopathic loss of the cell monolayer. Both PDX-4 (Panel C) and PDX-6 (Panel A) cell cultures, on the other hand, showed few syncytia. Productive infection was verified by the detection of more than 250 pg/mL of the HIV core antigen, p24, in supernatants from each of the infected cultures. The levels of infectious virus, in contrast, were much lower in the PDX-4 and PDX-6 cultures than in the parental HeLa clone 1022 culture. Cell lysates were prepared from each of the cell cultures on the fourth day post-infection and examined for the presence of gp160/gp120 by Western blot analysis as described in Example 5. Both gp160 and gp120 were detected in HIV-infected HeLa clone 1022 cultures, while no gp120 and only a faint band corresponding to gp160 was found in HIV-infected PDX-4 and PDX-6 cultures.

These results demonstrated that expression of α_1-PDX in HeLa clone 1022 cells conferred resistance in these cells to HIV infection. Moreover, the presence of p24 antigen in the cell supernatants of such cultures in the absence of large amounts of infectious virus indicates that expression of α_1-antitrypsin Portland in these cells inhibits the formation of infectious enveloped virus particles. These results have important implications for preventing HIV infection of vulnerable cells both *in vivo* and *in vitro*.

It should be understood that the foregoing disclosure emphasizes certain specific embodiments of the invention and that all modifications or alternatives

equivalent thereto are within the spirit and scope of the invention as set forth in the appended claims.

WHAT WE CLAIM IS:

1. A furin endoprotease inhibitor comprising an α_1-antitrypsin variant protein or peptide fragment thereof having an amino acid sequence comprising the amino acids -Arg-X-X-Arg-, wherein each X is any amino acid, corresponding to positions 355-358 of the native α_1-antitrypsin amino acid sequence.

2. The furin endoprotease inhibitor of Claim 1 that is α_1-antitrypsin Portland wherein the amino acid sequence of α_1-antitrypsin Portland at positions 355-358 of the native α_1-antitrypsin amino acid sequence is -Arg-Ile-Pro-Arg-.

3. A nucleic acid having a nucleotide sequence that encodes an α_1-antitrypsin variant protein or peptide fragment thereof, wherein the amino acid sequence encoded by the nucleic acid has the sequence -Arg-X-X-Arg-, where each X is any amino acid, corresponding to positions 355-358 of the native α_1-antitrypsin amino acid sequence.

4. The nucleic acid of Claim 3 that is α_1-antitrypsin Portland wherein the amino acid sequence of α_1-antitrypsin Portland encoded by the nucleic acid has the sequence -Arg-Ile-Pro-Arg- at positions 355-358 of the native α_1-antitrypsin amino acid sequence.

5. A recombinant expression construct comprising a nucleic acid having a nucleotide sequence encoding an α_1-antitrypsin variant protein or peptide fragment thereof, wherein the amino acid sequence encoded by the nucleotide sequence comprises the amino acids -Arg-X-X-Arg-, wherein each X is any amino acid, corresponding to positions 355-358 of the native α_1-antitrypsin amino acid sequence.

6. The recombinant expression construct of Claim 5 that is α_1-antitrypsin Portland wherein the amino acid sequence of α_1-antitrypsin Portland encoded by the nucleotide sequence comprises the amino acids -Arg-Ile-Pro-Arg- at positions 355-358 of the native α_1-antitrypsin amino acid sequence.

7. A recombinant expression construct comprising the nucleic acid of Claim 3, wherein the construct is capable of expressing α_1-antitrypsin Portland in a culture of transformed cells.

8. A cell culture transformed with the recombinant expression construct of Claim 5, wherein the transformed cell culture is capable of expressing α_1-antitrypsin Portland.

9. The cell culture of Claim 8 comprising bacterial cells, yeast cells, insect cells or mammalian cells.

10. A homogenous composition of matter comprising α_1-antitrypsin Portland produced by the cell culture of Claim 8.

11. A furin endoprotease inhibitor according to Claim 1 capable of blocking endoproteolytic activation of a bacterial toxin.

12. The furin endoprotease inhibitor of Claim 11 wherein the bacterial toxin is diptheria toxin of *Corynebacterium diptheriae*.

13. The furin endoprotease inhibitor of Claim 11 wherein the bacterial toxin is anthrax toxin of *Bacillus anthracis*.

14. A pharmaceutically acceptable composition comprising a therapeutically effective amount of the furin endoprotease inhibitor of Claim 11 and a pharmaceutically acceptable carrier or diluent.

15. A gene therapy delivery system for a nucleic acid encoding an α_1-antitrypsin variant comprising the recombinant expression construct of Claim 5 and genetic means for delivery and expression of the recombinant expression construct into the cells of an animal.

16. The gene therapy delivery system of Claim 15 wherein the α_1-antitrypsin variant is α_1-antitrypsin Portland.

17. A method of inhibiting bacterial infection of cells comprising contacting the cells with the furin endoprotease inhibitor of Claim 11.

18. A method of inhibiting viral infection of cells comprising contacting such cells with the gene therapy delivery system of Claim 15.

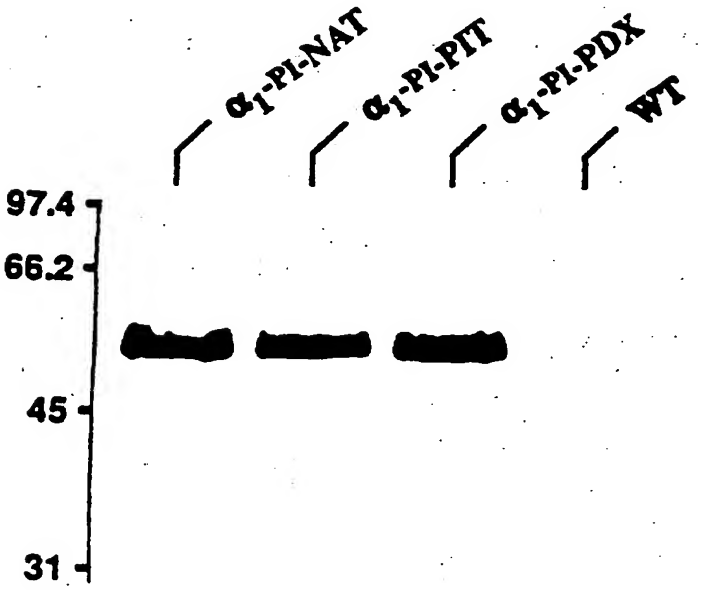
19. A method of inhibiting proteolytic processing of a biologically active protein or peptide in a cell comprising contacting such cells with the gene therapy delivery system of Claim 15.

20. The method of Claim 19 wherein the biologically active protein is pro-β-nerve growth factor, blood coagulation factor protein Factor IX, pro-

von Willibrand factor, complement factor C3 or renin.

21. A peptide having an amino acid sequence of 4 to 100 amino acids comprising the amino acid sequence -Arg-X-X-Arg-, wherein each X is any amino acid.

Figure 1



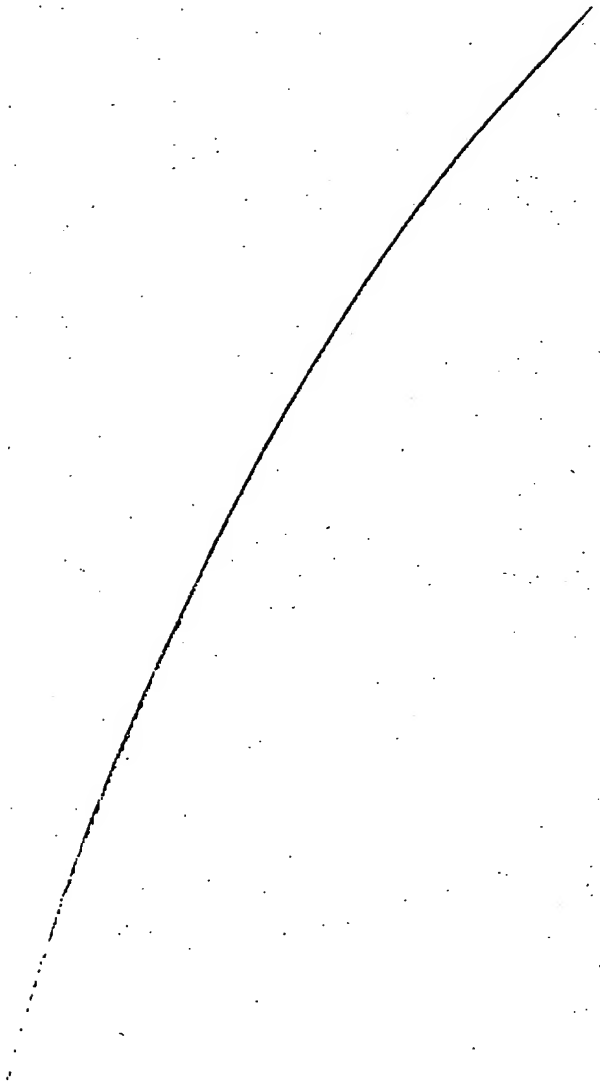
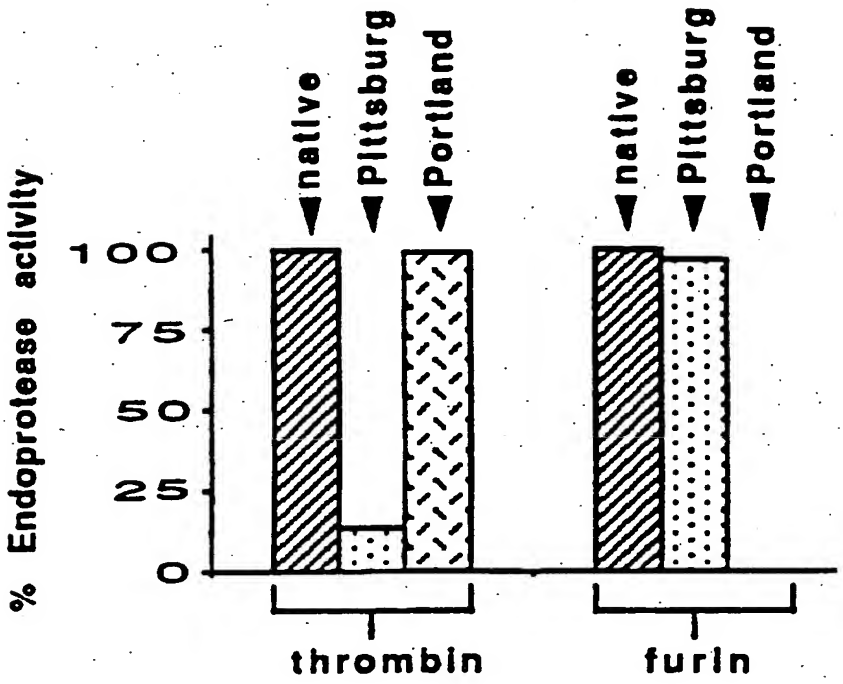
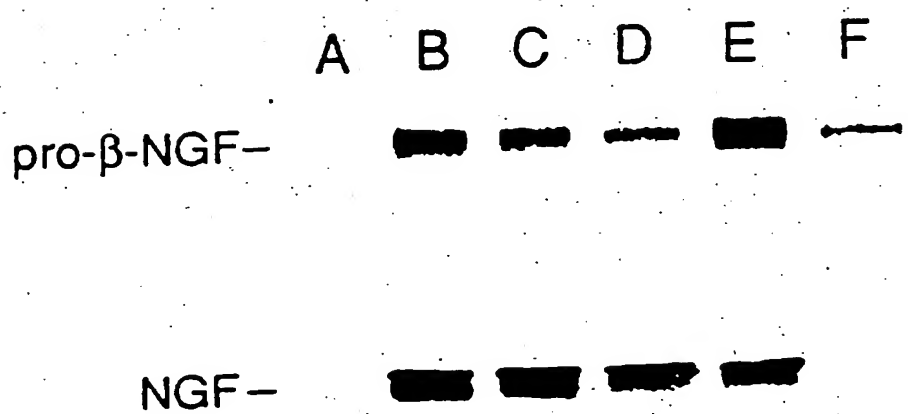


Figure 2



28.10.21

Figure 3



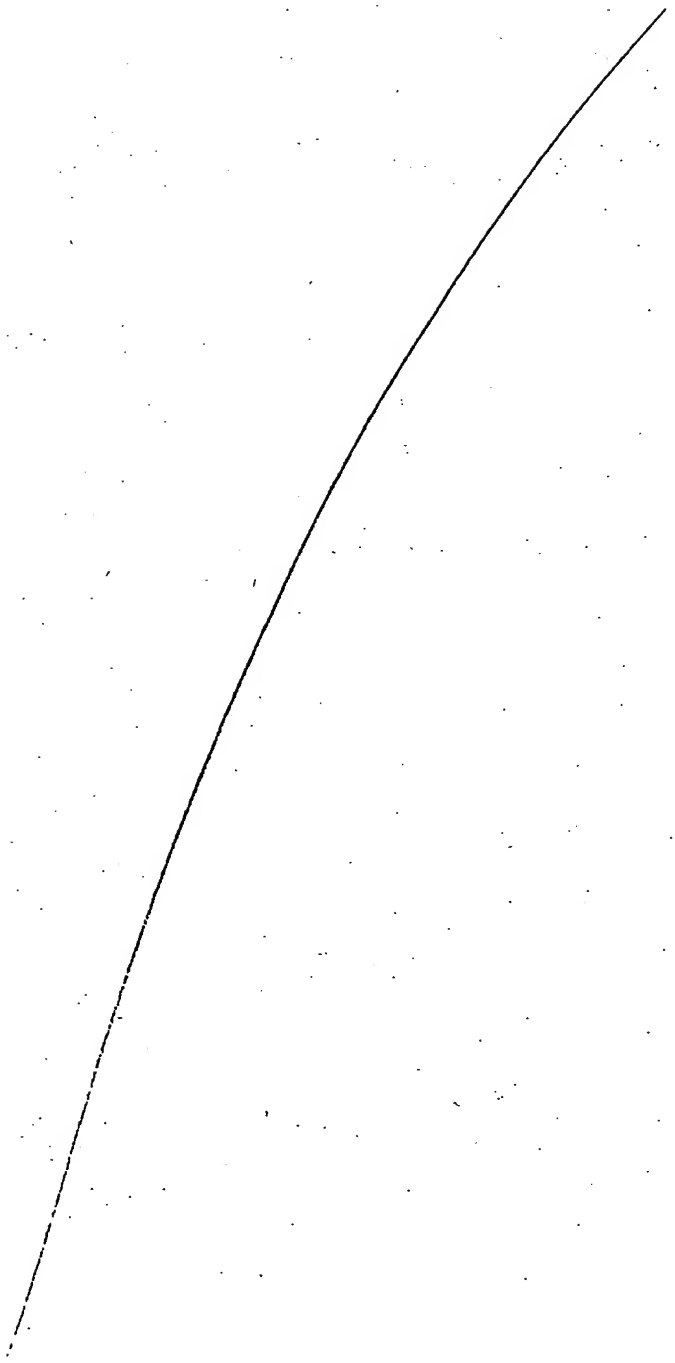
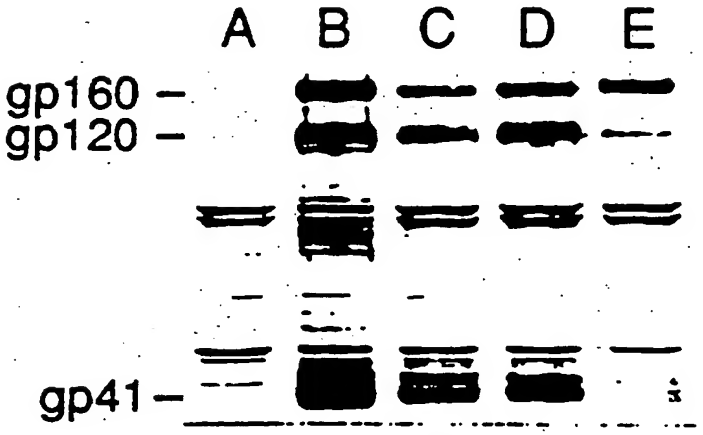
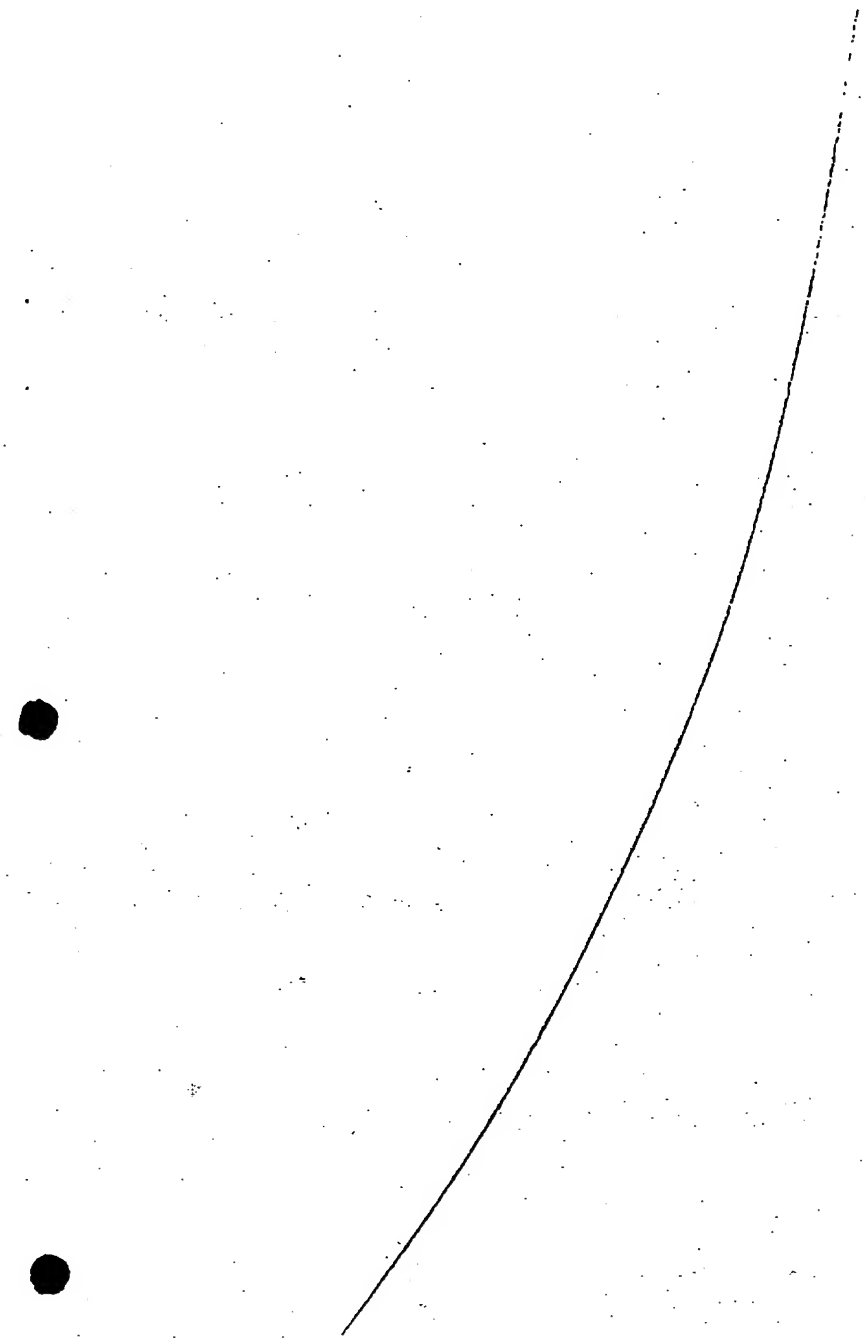


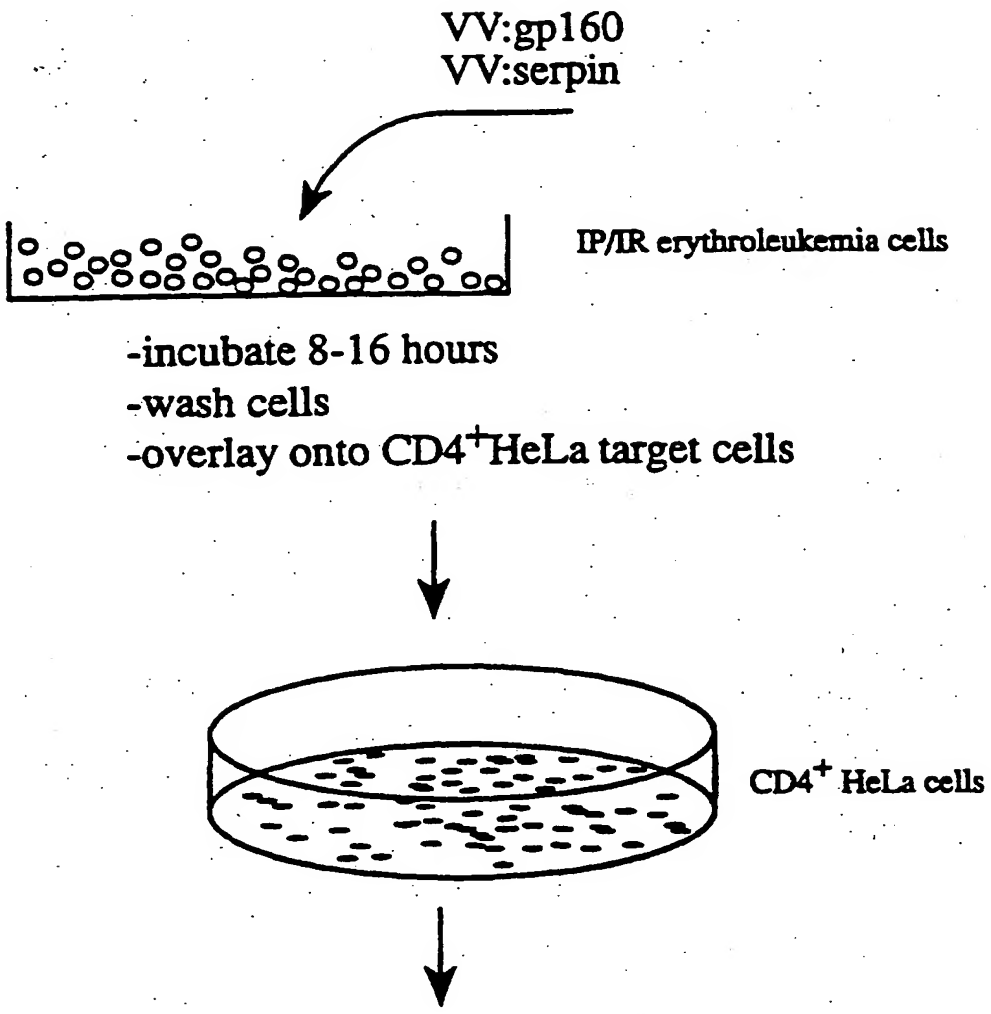
Figure 4

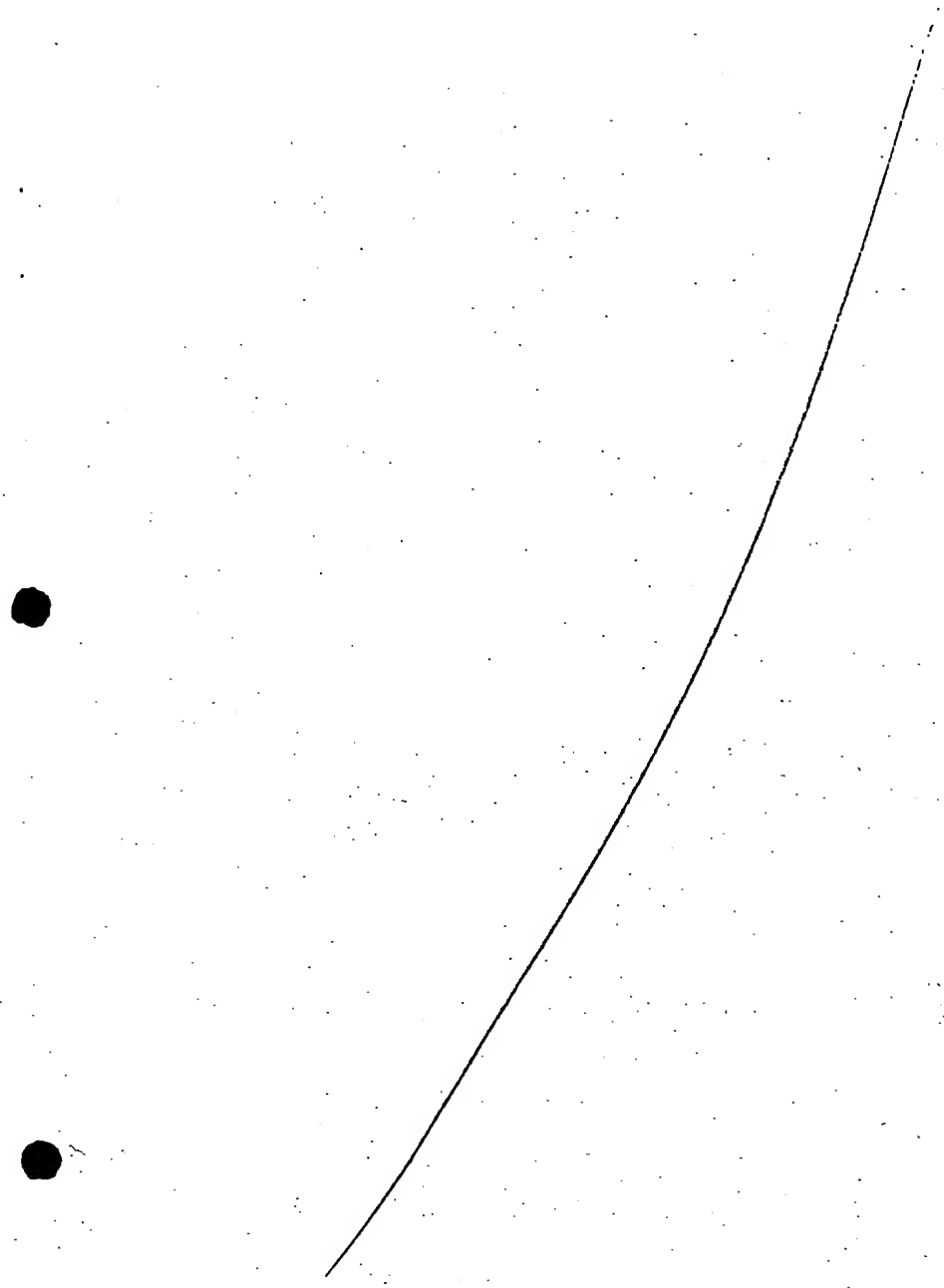




12·01·82

Figure 5


VV:gp160
VV:serpin
IP/IR erythroleukemia cells
-incubate 8-16 hours
-wash cells
-overlay onto CD4+HeLa target cells
CD4+ HeLa cells
-incubate 8-12 hours
-stain cells with crystal violet to detect syncytium formation



13:01:82

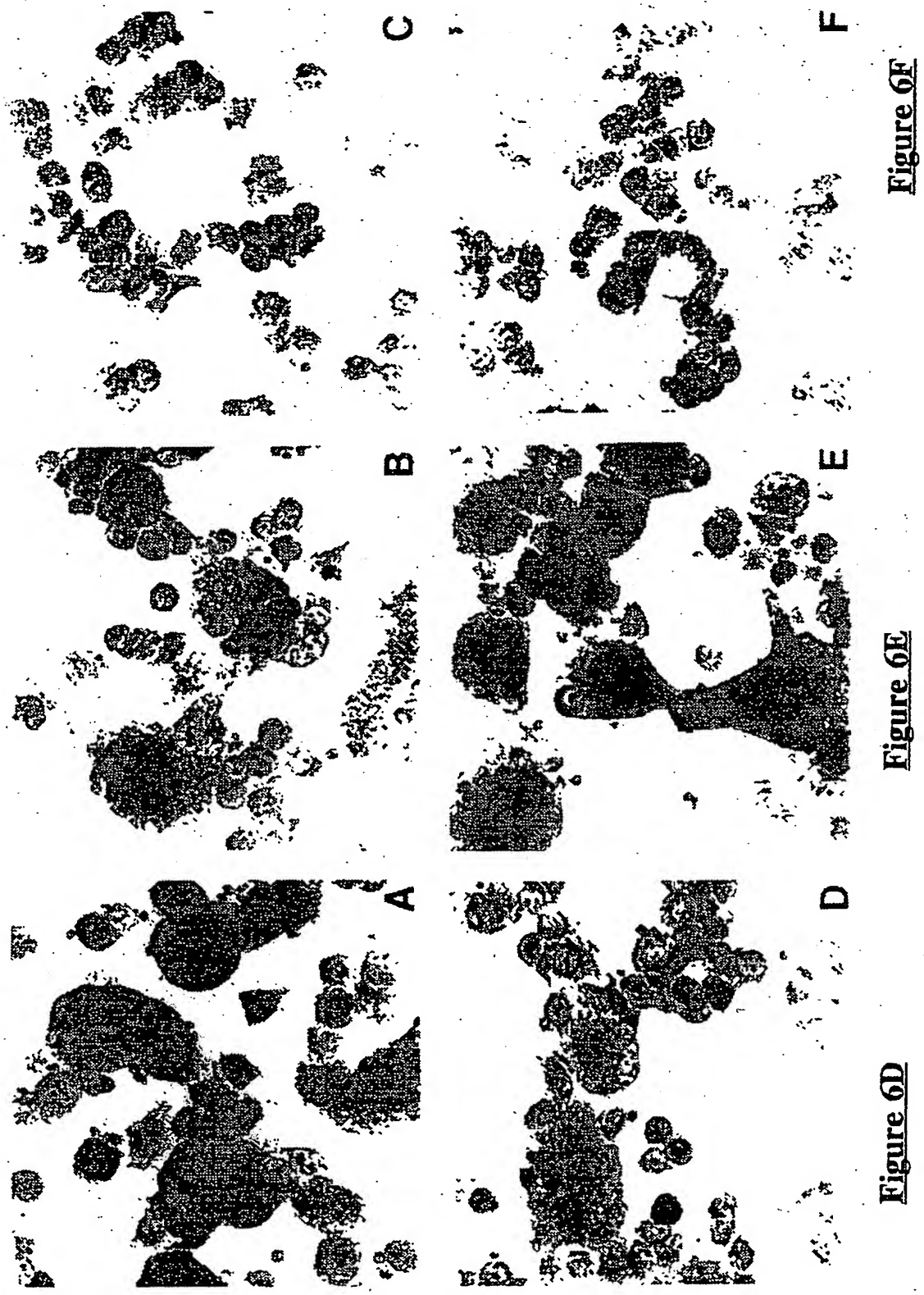


Figure 6A Figure 6B Figure 6C

Figure 6D Figure 6E Figure 6F

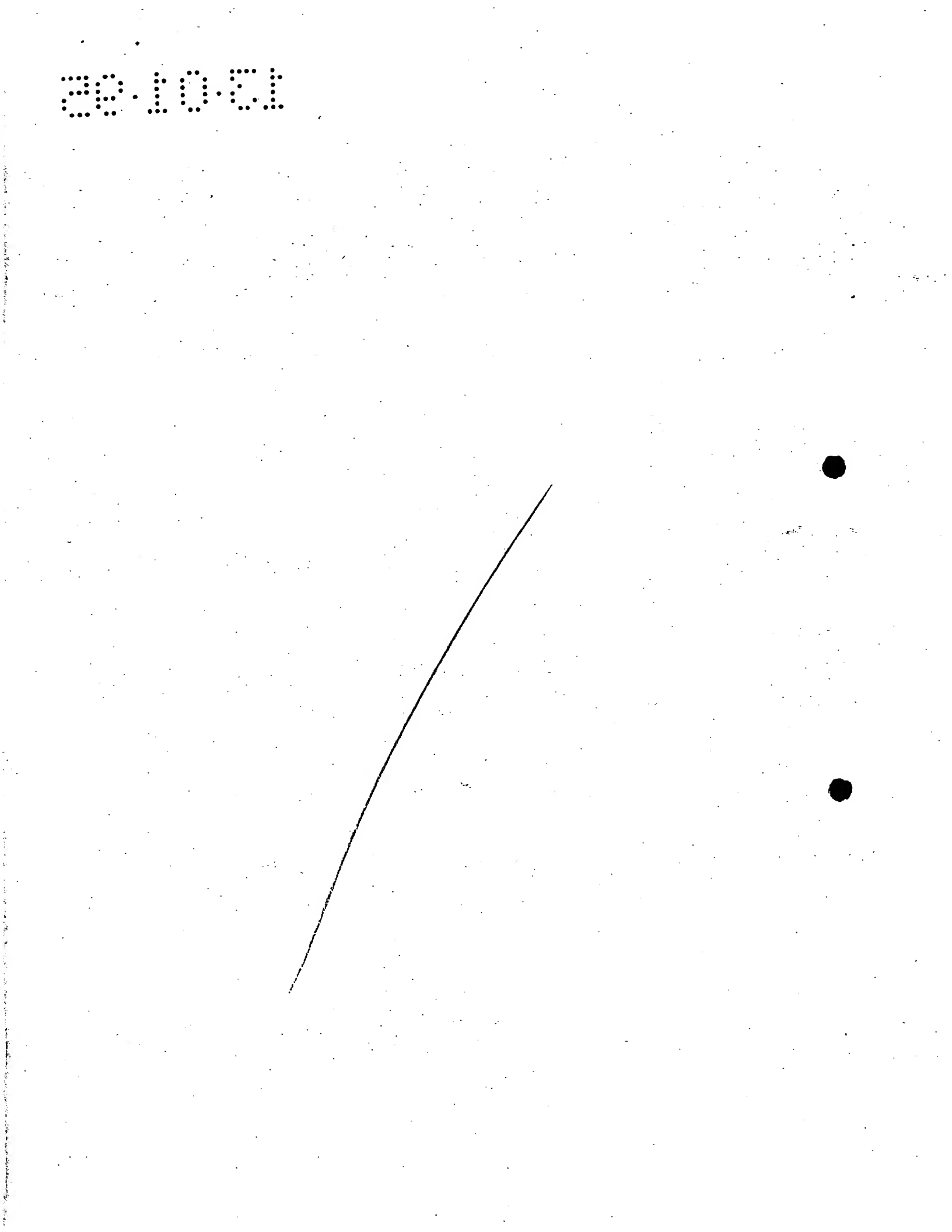

20.10.21

Figure 7A

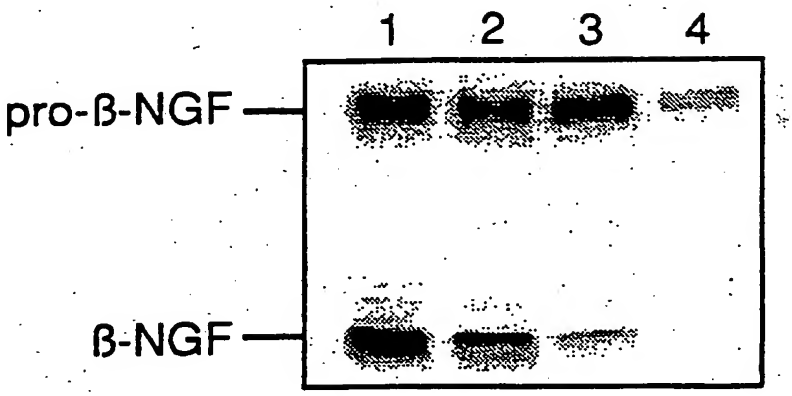
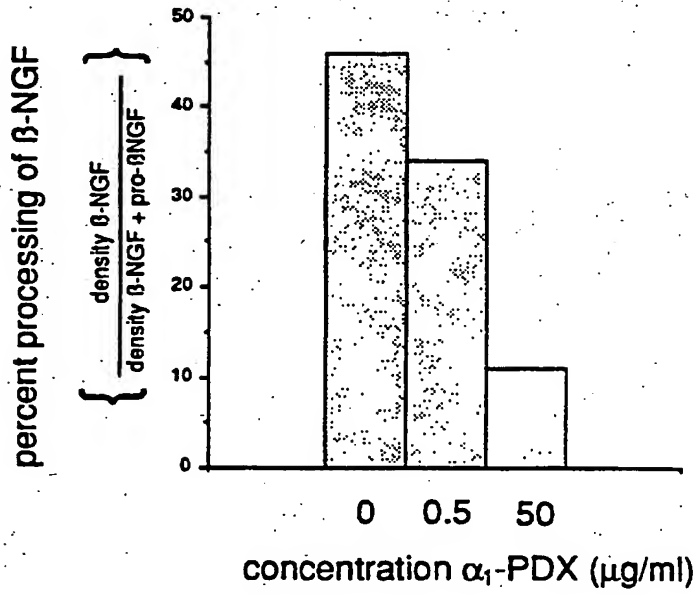
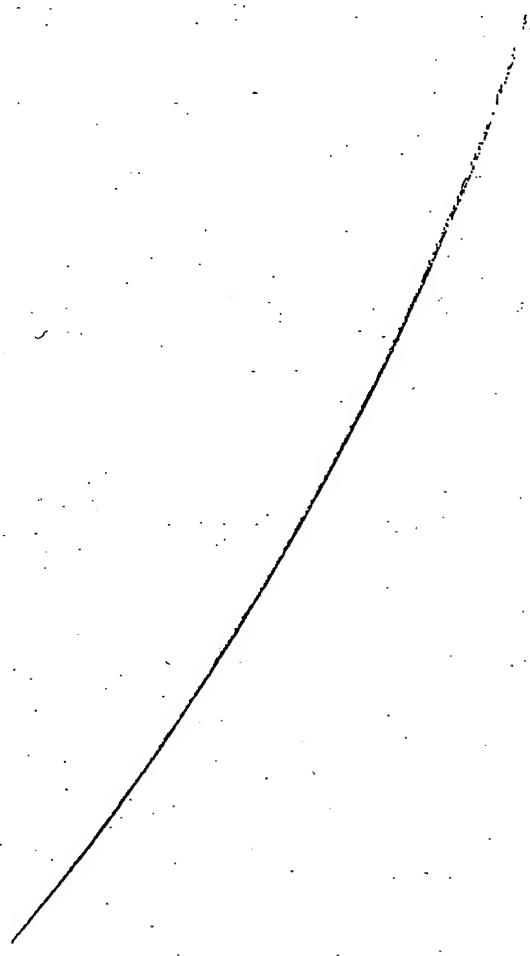


Figure 7B





13·01·82

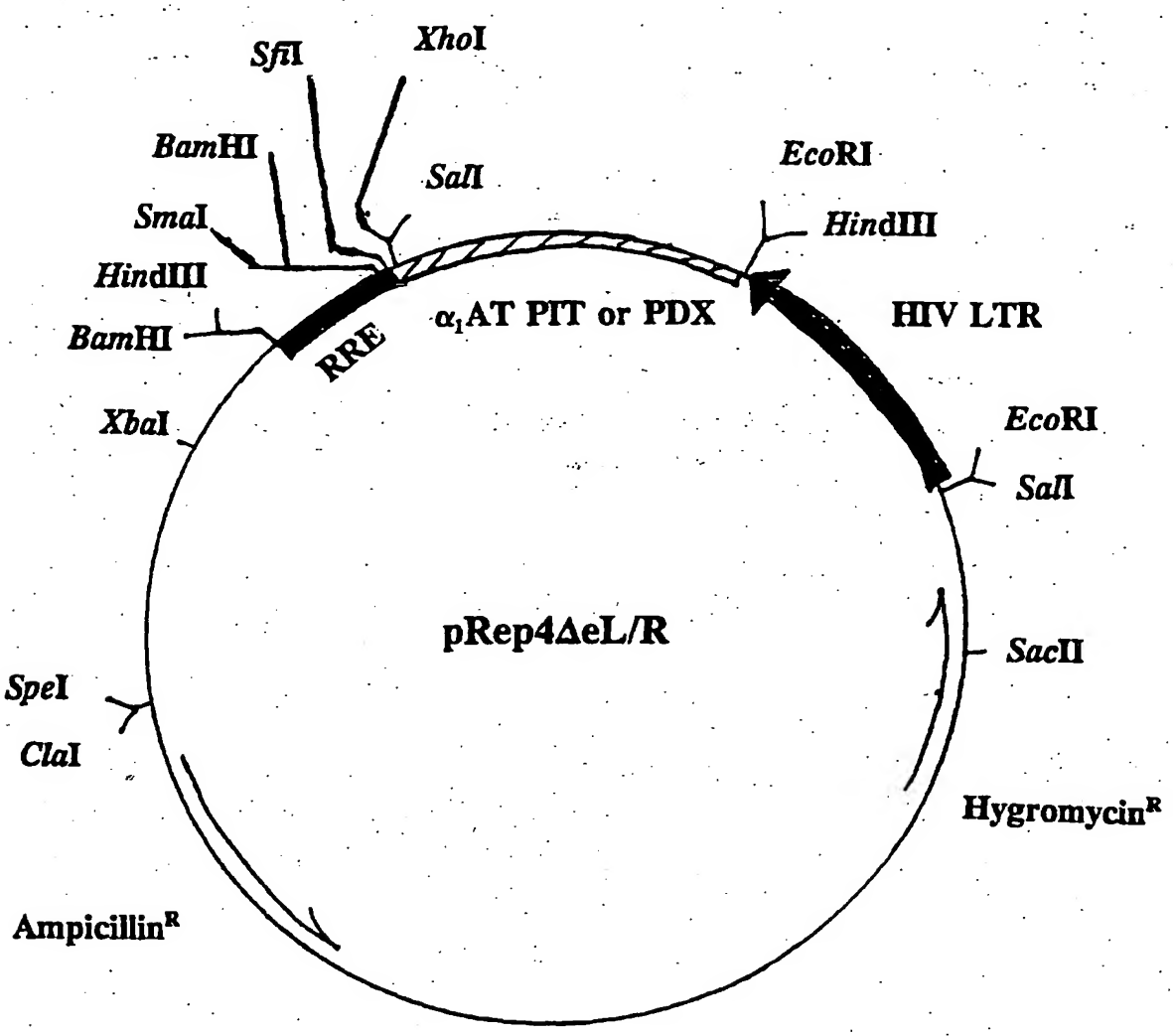
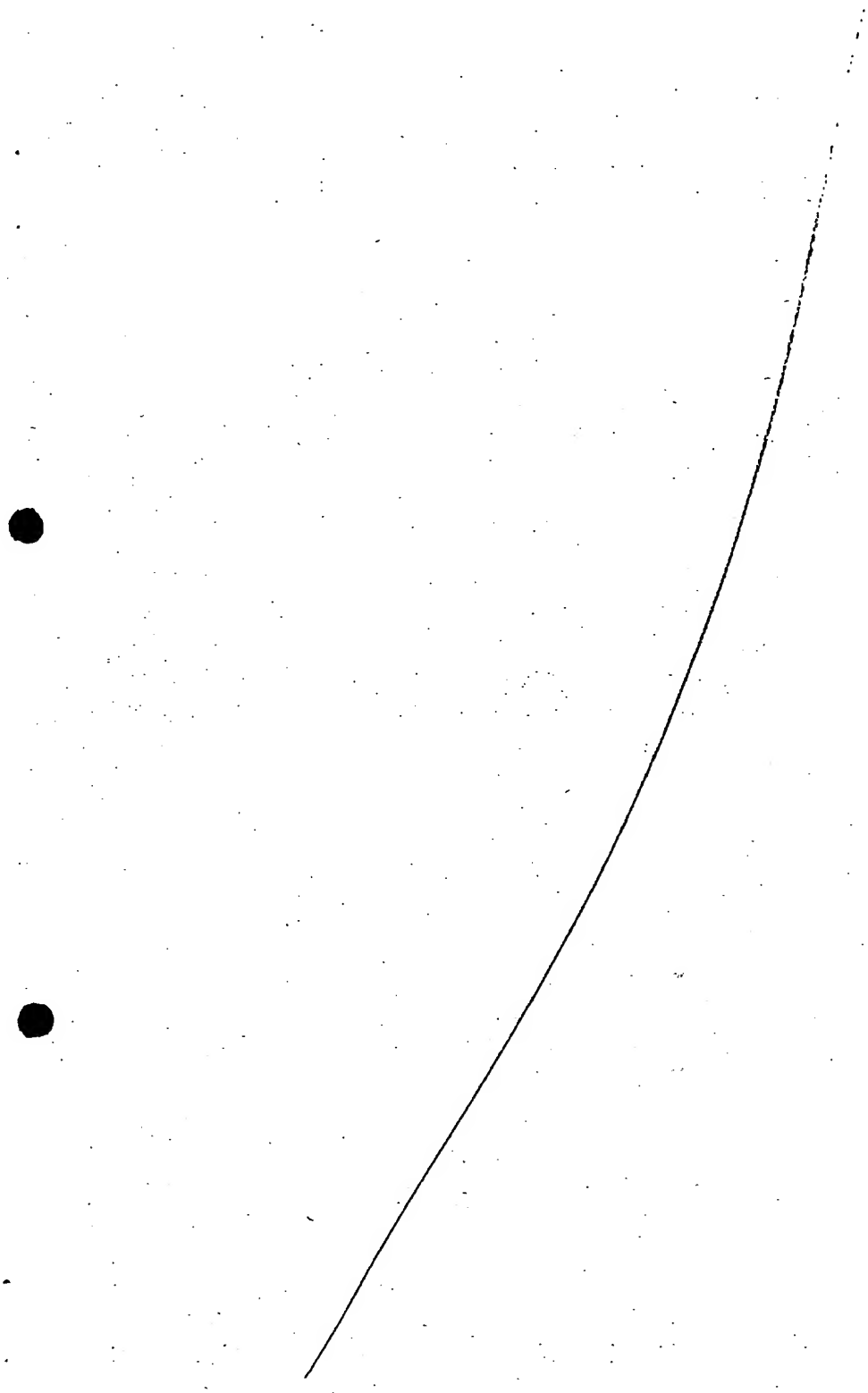

SfiI
XhoI
BamHI
SalI
EcoRI
SmaI
HindIII
HindIII
α1AT PIT or PDX
HIV LTR
BamHI
RRE
XbaI
EcoRI
SalI
pRep4ΔeL/R
SacII
SpeI
ClaI
Hygromycin^R
Ampicillin^R

Figure 8



12·01·82

Figure 9A

Figure 9B

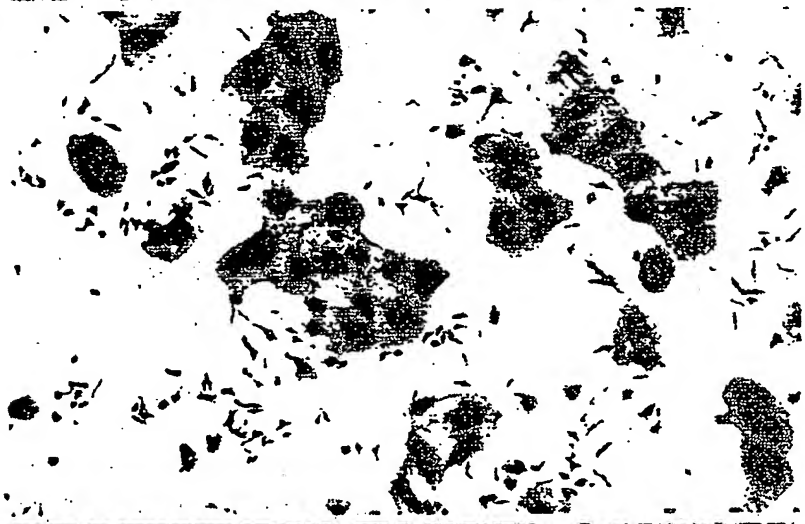


Figure 9C